Available online on www.ijppr.com
International Journal of Pharmacognosy and Phytochemical Research 2017; 9(9); 1260-1265
doi: 10.25258/phyto.v9i09.10314

ISSN: 0975-4873

Research Article

Toxicity Studies of Aqueous Extract of *Prismatomeris glabra*

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Received: 10th Aug, 17; Revised 30th Aug, 17, Accepted: 16th Sept, 7; Available Online:25th Sept, 17

ABSTRACT

This study was conducted to estimate levels for human consumption of an aqueous extract of *Prismatomeris glabra* (PG) roots. PG extract was prepared by boiling powdered roots for 10 minutes before drying in spray dryer. Cytotoxicity studies were conducted using cell lines from embryonic human liver (WRL68), liver cancer (HEP G2), colon cancer (HCT116), leukemic cells (K562) and mouse Leydig cells (CRL1714). Established method of comet assay was used to determine genotoxicity. Toxicity studies in mice were conducted for acute, subacute and subchronic effects. In all experiments, age-matched control mice were given normal saline. Gross necropsy biochemistry analyses were conducted following killing. PG showed no cytotoxic effects on WRL68, HEP-G2, K562 and CRL1714 cell lines. IC_{50} values in these cell lines were 2818, 2291, 1778 and 2400 µg/ml, respectively. PG also showed no effect on DNA. *In vivo*, mice were able to tolerate PG to a maximum single dose of 3 g/kg, p.o., 500 mg/kg/d, p.o., daily for 14 days, and at 100 mg/kg/d, p.o., daily for 3 months, respectively, without showing signs of toxicity or abnormal biochemical markers. In conclusion, based on toxicity data, PG root aqueous extract is generally safe for consumption.

Keywords: *Prismatomeris glabra*, toxicity, cytotoxicity, genotoxicity.

INTRODUCTION

The global market for herbal products has grown at an impressive rate due to global demand in traditional and alternative remedies[1]. The growth of herbal demand is associated with many reasons such as efficacy claims for alleviating symptoms of disease and presenting a cure, offering a safer treatment option than other therapies and providing more cost-effective[2]. However, issues of conservation, proper scientific research based on traditional knowledge, quality control and proper documentation are challenges to face within the advancement of herbal market[3]. Arising of a new herbal product may contribute to the market and industry sustainability. But this must be incorporated with rigid standards, safety issue and analytical approach through quality research[4]. Safety level for consumption of any natural product must be addressed and documented[5].

Toxicity studies attempt to identify and characterize adverse effects that develop in biological systems following exposure to a chemical or substance[6]. Toxicity studies on edible plants are important as some beneficial plants may contain toxic chemicals that adversely affect health[7]. Toxicity studies can be done *in vitro* and *in vivo*. *In vitro* toxicity studies include cytotoxicity and genotoxicity, these provide preliminary information on the toxicity of a substance or plant extract. Even though a positive correlation exists between *in vitro* and *in vivo* toxicity, the latter study needs to be conducted as it is difficult to determine possible target of toxic compound or plant extract in the whole organism without the right selection of *in vitro* model systems[8]. Furthermore, *in vivo* studies are needed to verify any finding from *in vitro* study even when the latter indicate non-toxic effect of test substance[9].

In *in vivo* study, animals are used to evaluate not only the safety level of a test substance but also the toxic effects in the whole organism, as animals are treated with various doses of test substance at different durations[10-12]. Guidelines for toxicity studies using animals are available such as Organization for Economic Co-operation and Development (OECD) guidelines provide standard methods that should be followed to produce reliable and harmonizable data. By using standard references such as Globally Harmonized System of Classification and Labelling of Chemicals[13], the use of animals as experimental subjects can provide guides for determining safety levels for human consumption of test substances[14].

A tropical plant, *Prismatomeris glabra* (PG), was claimed to have beneficial effects and improve wellness. A decoction of the plant root is traditionally used by Malay and indigenous people for various health purposes such as reducing lethargy, improving sexual desire, enhancing feeling of freshness and as illness remedies. PG normally grows on hillsides and ridges of tropical forests at altitudes up to 700 m. In our previous studies, PG root apparently has sufficient antioxidant capacity to enhance wellness[15], and was proved to improve physical performance[16]. In the present study, PG was investigated for any toxic effect as well as its safety level for consumption.

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MATERIALS AND METHODS

Preparation of PG aqueous extract

PG plants were collected from the tropical jungle in the Peninsular under supervision of jungle officers. The plants were verified by phytologists from Forest Research Institute of Malaysia (FRIM). The plant was given a voucher code of PT/UiTM/AS1 and kept in the Faculty's herbarium. Fresh roots of the plants were chipped into small pieces within 24-48 h of collection and dried at 45ºC in the oven for three days. Dry root chips were grounded to crude powder before every 100 g of them were boiled in 1 L of distilled water for 10 minutes. The suspension from the boiling process was filtered using filter paper. The filtrate was collected and dried using laboratory spray dryer (Büchi Mini Spray Dryer B-290). PG aqueous extract powder was kept in -20ºC freezer until use.

Cell growth and maintenance

Four different cell lines were used namely human normal liver cell (WRL68), human liver cancer cell (HEP-G2), human leukemic cell (K562) and mouse Leydig cell (CRL1714). All cell lines were supplied by American Tissue Culture Collection (ATCC). Cell growth and maintenance were performed according to manufacturer's protocol. WRL68, HEP-G2 and CRL1714 cell lines were growth and maintained in a flask containing MEM media. RPMI media was used for growth and maintenance of K562 cell lines. 10% fetal bovine serum (FBS) was used to supplement both media. All cell lines were placed and kept in incubator with set temperature at 37ºC with 95% humidity and 5% CO_2. Cells with more than 70% confluence were taken for subculture into another flask containing suitable media. Subculture work was done using fresh and sterile disposable apparatus in sterile fume cupboard. Media was changed every 3 days for optimizing cells' growth.

Cell viability test

Cytotoxicity testing was adopted from previous published methods[17-18] and manufacturer's protocol. Cultured cells with more than 70% confluence were used for platting process. For WRL68, HEP-G2 and CRL1714, trypsin EDTA was used to suspend the cells before the cells were centrifuged for 3 min. Supernatant was removed and the pellet containing cells was suspended several times. For cell count determination, 20ml of cells plus media was taken and mixed with trypan blue before putting them into the haemocytometer. The viability of the cells was determined by the trypan blue exclusion method. For plating work, suitable amount of cells plus media were pipetted into selected well of 96-well plate. The plate was then incubated at 37ºC for 24 hours before cells were treated with sample. PG extract was dissolved in distilled water at concentration of 50 mg/ml. Serial dilution of PG solution was treated with the cell lines.

An incubated 96-well plate that was platted with cells 24-hour earlier was used to treat with PG. 100µl media, blank (media plus media) or serial concentration of PG solution were pipetted into the wells accordingly in replication The plate was incubated at 37ºC, 95% humidity and 5% CO_2 for 24-hour in an incubator. After 24-hour of incubation, 20µl MTS was added into treated wells and were incubated under similar condition for another 2 hours. Formazan, a product from MTS reduction, was measured spectrophotometrically at 492nm using microplate reader. The assay concentration that caused 50% reduction in cellular viability, the IC_{50}, was determined and expressed in µg/ml. The value of IC_{50} was used to determine the cytotoxicity level of PG extract.

Genotoxicity - Comet assay

Comet assay was conducted according to established method[19]. A mixture of melting agarose and blood were placed and layered on a slide. The slide was leaved for a while until the layer was frozen. The slide was placed in the coupling jar containing lysing working solution and incubated in refrigerator for an hour. The slide was then placed on the electrophoresis tray and gently soaked with electrophoresis buffer for 20 min. Electrophoresis was switched on for 20 min at 25 volt and 300 mA. The slide was removed from the tray and cleansed using neutralizing buffer. After the slide dried, 30 µl of ethanol bromide was dripped on the slide and kept in refrigerator. DNA damage was determined using electron microscope.

Animals

Mus musculus mice were used as animal subjects for the toxicity study. The mice were supplied by certified pet company and were delivered at the age of 4-5 weeks. Prior to any treatment, the mice were placed in the animal room for acclimatization for at least 2 weeks. Mice were maintained under standard condition with 12 h - 12 h of light and dark cycle in the ventilated animal room with set temperature at 25ºC. Chow and water were given ad libitum to the animal. For acute, sub-acute and sub-chronic toxicity studies, mice that weighed 30-35 g and aged 7-8 weeks were randomly divided into 2 groups, controls that received 0.1 ml/10g p.o. 0.9 % normal saline and PG-treated group that received PG at respective dose.

Acute toxicity

Acute toxicity study was adapted from previous studies[20]. Ten mice per group were used in which PG-treated mice received 0.1 ml/10g p.o. of 300 mg/ml of PG extract, the possible highest concentration that was equivalent to dose of 3g/kg body weight. Observation over 24-hour was made and any behavioral and physical changes would be recorded. After 24-hour, all mice were killed by gentle cervical dislocation and blood was withdrawn by cardiac puncture technique and collected in glass tube. After 4 hours at room temperature, blood was centrifuged for serum separation. The serum was immediately sent for wet biomarkers for alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH), urea and creatinine using Roche-Hitachi 911 analyzer (Roche Diagnostics, Indianapolis, IN).

Sub-acute toxicity

Repeated dose of PG was conducted 14 days based on previous studies[7,21]. Eighteen mice were randomly divided into control (n=9) and PG-treated (n=9) groups. PG-treated groups received 500 mg/kg of PG (0.1 ml/10g p.o./day of 50 mg/ml PG dissolved in 0.9% normal saline). All mice were observed throughout 14 days for any physical and behavioral changes. On day 15, mice were sacrificed by gentle cervical dislocation. Blood was immediately

Table 1: Effect of different concentration of PG on WRL68, HEP-G2, CRL1714 and HCT116 cells viability. Data is expressed in mean ± SD.

PG concentration (µg/ml)	Cell viability (%)			
	WRL68 (n=3)	HEP-G2 (n=3)	CRL1714 (n=6)	HCT116 (n=3)
10	97.3 ± 1.5	87.5 ± 5.5	99.0 ± 6.0	92.7 ± 11.4
50	88.7 ± 11.8	87.7 ± 13.7	98.7 ± 6.2	88.7 ± 6.5
100	86.6 ± 11.9	82.0 ± 3.7	96.2 ± 0.6	81.8 ± 6.9
500	77.5 ± 8.5	77.2 ± 17.2	93.7 ± 5.8	76.1 ± 11.4
1000	69.4 ± 10.8	74.8 ± 6.6	88.0 ± 5.2	62.9 ± 6.8
5000	38.4 ± 28.8	21.7 ± 0.9	15.0 ± 7.7	27.3 ± 18.7
IC_{50} (µg/ml)	2818	2291	2400	1778

Table 2: Serum biomarkers of control and PG-treated mice at 24 h following acute treatment*.

Group	Body weight (g)	ALT U/L	AST U/L	LDH U/L	Urea mmol/L	Creatinine µmol/L
Control (n=10)						
Mean	30.9	63.2	128.6	709.7	6.9	46.0
S.D.	1.6	22.5	56.6	176.4	0.9	3.9
Range	28.6–33.5	34.4–114.0	64.5–261.6	349.9–955.0	5.9–8.5	40.0–51.0
PG-treated (n=10)						
Mean	31.4	60.5	168.1	889.2	7.2	43.5
S.D.	2.1	18.1	78.1	412.8	1.1	2.5
Range	28.7–35.4	40.9–99.2	74.6–345.8	152.5–1663.1	5.0–8.4	39.0–47.0
Unpaired t-test p value	0.507	0.770	0.212	0.222	0.505	0.110

*Control mice received 0.9% NaCl p.o. while treated mice received 3 g/kg body weight of PG extract, p.o. Values are expressed in mean±S.D.

withdrawn for serum biomarkers as explained above.

Another group of mice were divided into control and PG-treated groups (n=9/group) for shorter sub-acute toxicity (7 days) but received similar treatment as above whom blood was used for Comet assay.

Sub-chronic toxicity

Sub-chronic study was conducted based on method used in the previous studies[22-23]. Twenty healthy mice were randomly divided into 2 groups for controls (n=10) and treated (n=10) with PG at a dosage of 100mg/kg (0.1ml/10g p.o./day). After week 13, mice were sacrificed and blood was collected via cardiac puncture for serum biomarkers assay similar to explained above except additional of triglyceride and cholesterol.

Statistical analysis

Data were expressed as mean ± SD. Independent-samples t-test was used for selected comparisons between samples. Alpha value was set *a priori* at $p<0.05$.

RESULTS AND DISCUSSION

Cytotoxicity

Effect of PG on human liver (WRL68), human liver cancer (HEP-G2), mouse Leydig (CRL1714) and human colon cancer (HCT116) cell lines viability is shown in Table 1. At concentration of 10 µg/ml of PG, the cell viability was 97.3 ± 1.5 %. Increase of PG concentration at 50, 100, 500, 1000 and 5000 µg/ml reduced cell viability of WRL68 to 88.7 ± 11.8, 86.6 ± 11.9, 77.5 ± 8.5, 69.4 ± 10.8 and 38.4 ± 28.8 %, respectively. By plotting a smooth line on a graph, concentration of sample that inhibits 50% of cell population *in vitro*, or half maximal inhibitory concentration (IC_{50}), of PG on WRL68 was identified at 2818 µg/ml. Similar to WRL68, cell viability of HEP-G2, CRL1714 and HCT116 also decreased as concentration of PG increased. IC_{50} of PG on HEP-G2, CRL1714 and HCT116 was plotted at 2291, 2400 and 1778 µg/ml, respectively.

PG showed little cytotoxic effect on WRL68, HEP-G2, CRL1714 and HCT116 cell lines. Based on the classification of cytotoxicity by Abbas et al. (1994)[24], cytotoxic effect of aqueous extract of PG roots is poor. When these IC_{50} are extrapolated to estimate LD_{50} based on a regression formula published by Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) (2007)[25], LD_{50} values of PG become 2029, 1879, 1710 and 1912 mg/kg body weight from WRL68, HEP-G2, HCT116 and CRL1714 treatments, respectively. Based on classification of health hazard derived by GHS (2005)[13], PG is classified between category 4 and 5, which is low toxic.

Genotoxicity

The percentage of non-damaged DNA or head DNA of control group was 78.0 ± 13.2 % with 22.0 ± 13.2 % of control blood showing tail DNA (damaged DNA). For PG-treated group, percentage of head and tail DNA were 76.1 ± 15.1 % and 23.9 ± 15.1 %, respectively. There was no

Table 3: Serum biomarkers of controls and PG-treated following 14 days of daily treatment*.

Group	ALT U/L	AST U/L	LDH U/L	Urea mmol/L	Creatinine umol/L
Control (n=10)					
Mean	64.3	177.8	1255.0	6.6	42.9
SD	24.9	45.8	277.4	1.0	4.5
Min	38.6	132.0	679.0	5.5	35.0
Max	117.0	297.2	1638.9	8.7	49.0
PG-treated (n=9)					
Mean	77.8	194.5	1268.2	6.9	39.8
SD	33.7	97.0	244.1	1.2	1.8
Min	47.1	87.0	871.8	5.5	37.0
Max	145.3	395.6	1604.7	9.5	43.0
paired t-test					
p value	0.328	0.631	0.914	0.471	0.069

*Control mice received 0.9% NaCl p.o. while treated mice received 500 mg/kg body weight of PG extract, p.o. Values are expressed in mean±S.D.

significant difference of head and tail DNA between control and PG-treated groups. This means there was no evidence of DNA damage in blood of mice treated daily with PG (500 mg/kg) for 7 days. This is an important finding which indicates that PG is not mutagenic, thus is likely not carcinogenic. All mutagens are carcinogens even though not all carcinogens are mutagens[26-27].

Acute, sub-acute and sub-chronic toxicity study

Administration of PG at maximal dose of 3 g/kg body weight, p.o. to mice produced no fatality or abnormalities in behavior or other physical signs within 24 h of its administration. Mice treated with PG also did not show any sign for toxicity based on OECD 423 clinical signs for toxicity, viz. lethargy, tremor, aggression, loss of fur etc.[28]. There was no change of body weight within 24 h in both groups. Liver, lung, heart and kidneys appeared normal upon gross necropsy. Serum biomarkers of control and PG-treated mice at 24 h following treatment with a single dose are as shown in Table 2. There was no significant difference of ALT, AST, LDH, urea and creatinine between control and PG-treated groups.

None of the animals that were treated with PG (500 mg/kg body weight/day p.o., daily for 14 days) experienced fatality or showed abnormality of behavior or showed other physical signs or symptoms. Control and PG-treated mice had identical body weight prior to experiment. Both groups showed significant gain in body weight after 14 days. Serum biomarkers of sub-acute control and PG-treated mice are shown in Table 3. One PG-treated sample was not measured due to contamination. Similar to in acute study, ALT, AST, LDH, urea and creatinine of controls and PG-treated in sub-acute were also identical.

At week 2 of sub-chronic toxicity, one control mouse died from severe external injuries due to multiple fights with group members. There was no abnormality of liver, heart, kidney and lungs upon gross necropsy of this mouse as soon as the fatality was noticed. No fatality was recorded in other PG-treated mice throughout the study. Overall, there was no sign of abnormality and toxicity symptoms in mice from both groups throughout 13 weeks of study. Body weights of control and PG-treated were also similar throughout the study. Table 4 shows serum biomarkers of both groups following sub-chronic treatment. One PG-treated sample was excluded due to contamination. Also similar to acute and sub-acute studies, all biomarkers including triglyceride and cholesterol of sub-chronic control and PG-treated mice were not different.

PG roots are boiled and consumed as a traditional remedy to improve wellness and stamina and for its aphrodisiac effects. There is no report of adverse effect of PG amongst the people who consume decoction of the plant root. However, any traditional remedy requires toxicity study necessarily for determination of safe doses for administration and identification of toxic effects[11-12]. According to testimonials from villagers and indigenous people (orang asli), estimated amount of PG used for decoction process for consumption is about 30 – 40 g. Hence, a person with body weight of 70 kg consuming 35 g of PG obtained an equivalent human dose of 0.5 g/kg. In this study, boiling 100 g of PG roots in 1 L water yielded 3.5 g of spray dried aqueous extract that is 3.5% recovery. This means a test dose of 500 mg/kg body weight of aqueous extract in mice is equivalent to 14.3 g roots/kg, which is equivalent to more than 28 times the human dose. According to USEPA 2012 on safety factor, doses for experimental animals can be between 10 to 100 times the human doses[29]. The dose used for determination of acute toxicity in mice was 3 g/kg body weight which was equivalent to 171 times the human dose. For sub-acute and sub-chronic toxicity studies, doses used in mice were 500 and 100 mg/kg, respectively. The sub-chronic dose was 6 times greater than the human dose. Oral administration of all doses of PG to mice did not result in fatality and there were no signs and symptoms of toxicity in experimental animals. In fact, all mice given PG orally at different doses and for different durations of treatment showed normal behaviour and were even more active than their controls. Sub-acute administration of PG (500 mg/kg, p.o., every day for seven days) did not cause genotoxic effects as measured through comet assay. In cultured cell lines, PG did not cause cytotoxicity in normal and cancer cells.

Maximum solubility of PG was about 320 mg/ml. Administration of an oral dose of 3g/kg body weight of aqueous extract of PG roots to mice did not cause fatality

Table 4: Serum biomarkers of controls and PG-treated mice after 13 weeks of treatment*.

Control (n=9)	ALT U/L	AST U/L	LDH U/L	Urea mmol/L	Creatinine umol/L	Triglyceride mmol/L	Cholesterol mmol/L
Mean	68.7	191.4	1014.9	7.8	44.3	1.6	2.7
SD	12.3	93.9	323.7	1.1	4.0	0.5	0.8
PG-treated (n=9)							
Mean	53.4	149.8	771.9	7.4	41.3	1.4	2.7
SD	20.5	34.5	313.0	1.6	4.1	0.4	0.4
unpaired t-test							
P value	0.073	0.231	0.125	0.521	0.134	0.256	0.977

*Control mice received 0.9% NaCl, p.o. while treated mice received 100 mg PG /kg/day p.o. for 13 weeks.

and there were no behavioral abnormalities and no signs and symptoms of toxicity within 24 h of dosing. Acute dose of 3 g/kg PG p.o. was well tolerated by mice. For acute toxicity studies, oral doses of 2 and 5 g/kg were considered as maximum practical doses and used as dose limits[14]. Administration of higher than limit doses is not recommended as physiologically, it is not appropriate[30]. In mice given PG (3 g/kg, p.o.), there was no change in body weight within 24 h of dosing. Liver, lung, heart and kidneys appeared normal upon gross necropsy and were similar to those of controls. This was supported by AST and ALT as the enzyme markers for liver damage[31]. No difference in blood levels of these enzymes from control values and from normal range indicate no damage to liver of PG-treated mice. Other than no alteration in serum ALT and AST, a single dose of 3 g/kg PG also did not affect serum LDH, urea and creatinine at 24 h after dosing. Levels of urea and creatinine were used to evaluate renal function as both metabolites are protein derivatives which are excreted by the kidney[32]. Levels of these marker molecules were all in the range of normality.

GHS (2003) classification states when an oral dose that kills 50% of test animals (oral LD_{50}) is more than 2000 mg/kg, the test substance is classified as category 5 i.e., of low toxicity[33]. Since aqueous extract of PG roots was used in an acute dose of 3 g/kg, which was at its maximum solubility, and there were no fatality or abnormal signs and symptoms, the extract is safe in acute dosing, i.e. of low toxicity[34]. Repeated administration of highest dose may provide different outcomes, but is not proposed by any safety procedure for a test chemical[35].

Dose of PG extract used for the acute study was equivalent to 171 times the human dose. A margin of safety of 100 is typically used when extrapolating doses from animals to humans[36]. Intraspecie variation is given a factor of 10 and another factor of 10 for interspecies variation[37]. Considering mice are more susceptible to oral toxicity than humans[35], doses of PG decoction consumed by villagers are often low which may account for no adverse effects of PG in these people.

In conclusion, PG was proven safe when mice were able to tolerate PG to a maximum dose of 3 g/kg, p.o. without showing signs of toxicity over a 24-hour observation period. Mice that were sub-acutely and sub-chronically administered with PG (500 mg/kg/d, p.o., daily for 14 days or at 100 mg/kg/d, p.o., daily for 3 months, respectively) did not exhibit signs of toxicity. Serum levels of ALT, AST, creatinine, urea and lactate dehydrogenase were unchanged by PG. PG also had no effect on DNA as measured by comet assay and did not produce damage to organs as determined by gross necropsy. PG also showed no cytotoxic effects on selected cell lines. Thus, PG root aqueous extract appears to be safe for human consumption.

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